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LRP6 Mutation in a Family with Early Coronary Disease and Metabolic Risk Factors

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Abstract

Coronary artery disease (CAD) is the leading cause of death worldwide and is commonly caused by a constellation of risk factors called the metabolic syndrome. We characterized a family with autosomal dominant early CAD, features of the metabolic syndrome (hyperlipidemia, hypertension, and diabetes), and osteoporosis. These traits showed genetic linkage to a short segment of chromosome 12p, in which we identified a missense mutation in *LRP6*, which encodes a co-receptor in the Wnt signaling pathway. The mutation, which substitutes cysteine for arginine at a highly conserved residue of an epidermal growth factor–like domain, impairs Wnt signaling in vitro. These results link a single gene defect in Wnt signaling to CAD and multiple cardiovascular risk factors.

Coronary artery disease (CAD) due to atherosclerosis results in myocardial infarction (MI) and is the leading cause of death worldwide (1). Epidemiologic studies and clinical intervention trials have established the key roles of specific risk factors for CAD, including smoking, hypertension, high low-density lipoprotein (LDL) cholesterol, high triglycerides, low high-density lipoprotein (HDL) cholesterol, and diabetes mellitus (2–4). Surprisingly, many of these risk factors cluster with one another more often than expected by chance (5,6). This metabolic syndrome is recognized to be a common cause of CAD; however, the molecular mechanisms that unify their association have been obscure.

The marked increase in risk of early cardiovascular mortality to a second monozygotic twin when the first has died from early CAD provides evidence for a strong genetic effect and supports investigation of families with early disease (7). Such studies have the capacity to

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Supporting Online Material

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Materials and Methods

Figs. S1 to S3

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identify genes and pathways whose altered function impart large effects on CAD outcome; these may provide insight into basic mechanisms that are also involved in common forms of disease and that may be manipulated for health benefit.

From a screen of patients and families with CAD, we identified one extreme outlier kindred with an extraordinary prevalence of early CAD. Kindred CAD-100 is of Iranian ancestry, ascertained via Subject II-7 (table S1), who presented with MI at age 48. CAD risk factors included hypertension, hyperlipidemia, and diabetes mellitus; he had never smoked and his body mass index (BMI) was 24. Evaluation revealed critical stenosis of all three major coronary arteries, which led to coronary artery bypass grafting. His course was complicated by progressive atherosclerosis of the grafts and internal carotid arteries. At age 62, he suffered a low-impact hip fracture and was found to have very low bone mineral density of unknown cause (z score of -3.4 at the femoral neck of his intact hip). He died from a stroke at age 72.

Among 58 blood relatives of the index case, 28 were diagnosed with early CAD (MI, angina, or sudden cardiac death) at or before age 50 (men) or 55 (women) (Fig. 1). Of these, 23 have died from CAD (mean age of death, 52 years). In contrast, kindred members without early CAD died at a mean age of 81. This familial clustering is noteworthy given that early CAD and early CAD death are uncommon in the general population (8,9).

Detailed clinical data were obtained for all available kindred members, including 13 affected with early CAD, 5 free of early CAD at or beyond the age threshold of 50 years (men) and 55 (women), and 9 younger asymptomatic members (CAD phenotype unknown; mean age 35 years) (table S1). Cardiac risk factors before or at presentation among affected subjects were surprisingly homogeneous, including high fasting LDL cholesterol in all (mean 176.4 mg/dl, $nl < 130$ mg/dl), high fasting triglycerides in 90% (mean 240 mg/dl, $nl < 150$ mg/dl), marked hypertension in all (mean 175/103 mm Hg, $nl < 140/90$, typically diagnosed after age 40), and type II diabetes mellitus in 77% (fasting blood glucose > 126 mg/dl; typically diagnosed after hypertension and hyperlipidemia). Despite high triglycerides, HDL levels were normal in all, and only one had a history of smoking. Nearly all of the affected subjects met criteria of the NIH National Cholesterol Education Program for metabolic syndrome based on the presence of diabetes, high triglycerides, and hypertension (10). Although obesity is strongly associated with metabolic syndrome and each of these risk factors, it is noteworthy that obesity is absent among affected subjects (mean BMI 24.6, none greater than 26). In contrast to these affected subjects, the five unaffected family members all had normal levels of blood pressure (mean 116/81 mm Hg), LDL cholesterol (mean 97.8 mg/dl), and triglycerides (mean 60.75 mg/dl), and type 2 diabetes mellitus was absent. Finally, among younger subjects with CAD phenotype unknown, all nine had high LDL levels and seven had high triglyceride levels, whereas hypertension and glucose intolerance were less frequent (table S1).

The marked clustering of early CAD and risk factors in this kindred suggests a strong genetic component. Among sibships in which all subjects are beyond the age threshold for onset of CAD, the offspring of single affected parents yielded 17 affected and 15 unaffected subjects, and male-to-male transmission is present; in addition, the union of two affected first cousins yielded six affected and one unaffected offspring. Similarly, LDL levels are strongly bimodal in the kindred, with all members having either high levels (157 to 195 mg/dl) or low levels (92 to 105 mg/dl), and these levels cosegregate with early CAD. This extreme familial clustering and segregation of phenotypes within the kindred is unlikely to be explained by chance or multifactorial determination and provides strong evidence that early CAD is transmitted as a highly penetrant autosomal dominant trait.

Nineteen family members were available for genetic studies, including seven with early CAD, five unaffected subjects, and seven with CAD phenotype unknown due to young age. A

genome-wide analysis of linkage was performed using Affymetrix 10K Gene Chips. We analyzed linkage using all single-nucleotide polymorphisms (SNPs) by applying two pre-specified models of the trait locus—a conservative model that specified 90% penetrance, 1% phenocopies, and disease allele frequency of 0.001 and a stringent model that specified 99% penetrance, 0.1% phenocopies, and allele frequency of 0.0001 (11). Results under both models demonstrated significant evidence of linkage to a segment of chromosome 12p, and no other interval yielded a logarithm (base 10) of the odds ratio (lod score) greater than 1.5. Under the stringent model, the maximum multipoint lod score was 4.4 for linkage of CAD within the 2.7-cM interval flanked by loci rs2213177 and rs747726 (Figs. 1 and 2A; odds ratio 25,000:1 in favor of linkage). Results of linkage under the conservative model were similar. Linkage was confirmed by genotyping 11 highly polymorphic di- and tetranucleotide repeat markers this interval of 12p (Fig. 1), and virtually indistinguishable results were obtained when all markers were combined in the analysis. The observed lod scores approximate the theoretical maximum under the specified models. It is worth noting that the index case, who was the offspring of affected first cousins, was homozygous across this interval, implying that he was homozygous for the underlying disease-causing mutation.

The lod-1 interval spans only 750,000 base pairs and contains only six annotated genes: *ETV6*, *BCL2L14*, *LRP6*, *MANSC1*, *LOH12CR1*, and *DUSP16* (fig. S1). Among these, *LRP6* (LDL receptor-related protein 6) is noteworthy. *LRP6* and its close paralog *LRP5* serve as co-receptors with frizzled proteins (members of the G protein-coupled receptor family) for Wnt ligands (12). Mice deficient for *LRP5* develop hypercholesterolemia and impaired glucose tolerance on a high-fat diet (13), and LDL levels are markedly increased on the apolipoprotein E-deficient background (14). In addition, deficiency for either *LRP5* (in the human and mouse) or *LRP6* (in the mouse) results in early severe osteoporosis (15,16). This latter observation recalls the unexplained osteoporosis of the index case. Evaluation of kindred members identified two additional affected males with early hip fractures (ages 48 and 68) and low bone density in the three additional affected subjects studied; in contrast, bone density was normal in the one unaffected subject studied (table S1).

These findings motivated further evaluation of *LRP6*. Direct sequencing of all exons and intron-exon boundaries of *LRP6* in the index case revealed a single variant, a homozygous mutation that introduces a missense substitution, R611C (Fig. 2B). R611 lies in an epidermal growth factor (EGF)-like domain (fig. S2) and is conserved among *LRP6* orthologs ranging from *Xenopus* to human (Fig. 2C); it is also found in mammalian *LRP5*, *LRP2*, and *LRP3*. This mutation precisely cosegregated with early CAD in the kindred, was absent among 400 unrelated Iranian and 3600 U.S. Caucasian control chromosomes, and is predicted to be deleterious by the PolyPhen and Sift programs. Sequencing of the other five genes in the lod-1 interval identified no other missense or splice site mutations.

We next considered the impact of this mutation on CAD risk factors. Analysis revealed complete linkage of *LRP6*_{R611C} and high LDL, with a lod score of 5.5 (Fig. 2A, odds of 316,000:1 in favor of linkage). The difference in mean LDL levels between mutation carriers and noncarriers is significant (170 ± 12 mg/dl versus 98 ± 5 mg/dl, $P = 6 \times 10^{-6}$; Table 1). Because high LDL levels are found in all mutation carriers, regardless of age, this trait can serve as a biomarker of the mutation in subjects too young to manifest CAD. Similarly, *LRP6*_{R611C} imparts significant effects on triglyceride levels, blood pressure, fasting blood glucose, and prevalence of diabetes (Table 1). No significant effects were seen on HDL levels or body mass index. Finally, all five mutation carriers studied have low bone densities, each with values expected in less than 12.5% of the population ($P < 0.001$).

The functional significance of this *LRP6* mutation was explored by expression in NIH3T3 cells (17) (Fig. 3). In these cells, expression of *LRP6* potentiates Wnt signaling, assayed as LEF-1

mediated expression of luciferase. In the absence of added Wnt 3a, LRP6_{R611C} showed a 49% reduction of induced signaling compared with that of wild-type LRP6 ($P < 0.01$). The addition of low doses of Wnt 3a also showed markedly reduced signaling with LRP6_{R611C} (42% reduction, $P < 10^{-5}$). At high doses of Wnt 3a, however, Wnt signaling through LRP6_{R611C} does not differ significantly from that of the wild type ($P = 0.48$). Measurement of total LRP6 expression by Western blotting and cell surface expression measured by specific binding to Dkk-1 demonstrate similar levels of wild-type and mutant LRP6 (fig. S3, A and B). These findings are consistent with an impaired biochemical function of LRP6_{R611C}.

Our findings establish a causal link between *LRP6* mutation and early CAD with high LDL, high triglycerides, hypertension, diabetes, and low bone density. The evidence includes strong a priori evidence of segregation of the disease as an autosomal dominant trait in this kindred, linkage of this trait and underlying risk factors to a single small genomic interval, identification of a single rare mutation in the linked interval that alters a highly conserved amino acid, biochemical evidence that the mutation impairs function of the encoded protein, and evidence from mouse models that mutations in orthologs and paralogs of the identified gene confer similar effects on risk factors. We anticipate that the mutation we identified is very rare in the population; to date we have found no other suggestive mutations in *LRP6* among 400 unrelated subjects with CAD. Nonetheless, as for other Mendelian forms of important medical traits—including cardiovascular risk factors, neurodegenerative diseases, and pain perception, among many others (18–20)—the findings from this rare kindred may provide key insight into pathways that cause disease and that may be manipulated for health benefit. These findings motivate further investigation of the genes and activity of this pathway to search for inherited or acquired variation in Wnt signaling in common forms of CAD and metabolic syndrome.

The impact of the *LRP6* mutation on multiple CAD risk factors is notable. Most mutation carriers over age 45 meet criteria for the metabolic syndrome. Although the observed effects on these risk factors are substantial, they are not individually as large as those seen with many previously defined Mendelian traits. For example, the mean LDL levels among *LRP6* mutation carriers are 170 mg/dl, versus a mean level of about 300 mg/dl among carriers of heterozygous loss-of-function mutations in the LDL receptor (21). Similarly, the hypertension and diabetes in this family typically appear in middle age, at ages that could easily be mistaken for “garden variety” forms of these risk factors. These observations suggest that it is the combined effects of these risk factors that account for the very high cardiovascular risk to mutation carriers.

Because loss-of-function mutations in *LRP5* (12,15) and *LRP6* (16) result in reduced bone density, the osteoporosis among LRP6_{R611C} carriers lends further support to the functional significance of this mutation. Moreover, recent epidemiologic studies have found strong association of osteoporosis and CAD (22). Our observations suggest that osteoporosis and CAD can be pleiotropic consequences of impaired Wnt signaling, raising the question of whether the co-occurrence of osteoporosis and CAD might commonly identify individuals with inherited or acquired impairment in Wnt signaling.

Our findings underscore emerging evidence implicating effects of altered Wnt signaling on cardiovascular risk factors. Common intronic variants in the Wnt-responsive transcription factor *TCF7L2* result in altered insulin secretion and type II diabetes mellitus (23). Similarly, rare mutations in other Wnt-related transcription factors cause maturity onset diabetes of youth (24,25). The LRP6_{R611C} mutation confers effects not only on many risk factors but on CAD outcomes as well. The ubiquitous expression of *LRP6* (26) supports the possibility of pleiotropic effects in diverse tissues. Further investigation of Wnt signaling in patients with early CAD, metabolic syndrome, and its components may provide new insight into disease pathophysiology and approaches to prevention of these disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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27. Materials and methods are available as supporting material on Science Online.
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Fig. 1.

Relationships of members of kindred CAD-100 are shown. The index case is indicated by the arrow. Numbered individuals correspond to those in table S1. Individuals with early CAD are indicated by black symbols; individuals without CAD who are beyond age 50 (men) or 55 (women) are shown as unfilled symbols; and individuals who are without symptomatic CAD, are below these ages, and have high LDL levels (range 157 to 192) are shown as half-black, half-gray symbols. Individuals who were not studied are indicated by symbols with dots. Circles represent females; squares represent males. Symbols with a slash through them indicate deceased subjects. Genotypes of informative microsatellite and SNP markers are shown in their chromosomal order below the symbol for each individual and their distance in centimorgans and megabases from 12pter is indicated. Segments of the haplotype seen in the index case which segregate with CAD and/or high LDL levels are indicated by shaded boxes. The presence of the wild-type *LRP6* or *LRP6*_{R611C} mutation is indicated by a plus sign or a red asterisk, respectively. Subjects III-6, III-7, and III-8 are offspring of a homozygous mutation carrier and hence obligate mutation carriers; subjects III-3, III-4, and III-5 are also statistically likely to

be offspring of a homozygous carrier. This explains the high proportion of mutation carriers in generation III.

Fig. 2.

Mutation in *LRP6* cosegregates with early CAD and high LDL. (A) Multipoint lod scores for linkage of early CAD and high LDL cholesterol to 12p. Multipoint lod scores for linkage of early CAD (solid line) or high LDL cholesterol (dashed line) to proximal 12p are shown under the stringent model of the trait locus (27). Lod scores were calculated with the use of all chromosome 12 SNP data from Affymetrix 10K chips (27). SNPs tightly linked to the location of the maximum lod score are indicated and the location of *LRP6* is shown. The lod score peak occurs at zero recombination with marker *rs958812* and the lod-1 interval spans 2.7 cM for both traits. (B) The DNA sequence of a segment of *LRP6* exon 9 is shown from an unaffected kindred member (left), a heterozygous mutation carrier (middle), and the homozygous index case (right). A single base substitution (asterisk) changes the wild-type cytidine to thymidine leading to substitution of cysteine for arginine at codon 611. (C) A portion of the amino acid sequence of the second EGF-like domain of *LRP6* is shown from diverse vertebrate species. This segment is highly conserved and arginine is completely conserved from *Xenopus* to human.

Fig. 3. LRP6_{R611C} impairs Wnt signaling. NIH3T3 cells were transfected with plasmids encoding wild-type (WT) or mutant hemagglutinin-tagged LRP6 and Wnt reporter genes and incubated with indicated concentrations of purified Wnt 3a protein followed by an assay of Wnt signaling (LEF-1-dependent expression of luciferase) (27). The results are shown as mean and standard error of the mean of quadruplicate experiments. RLU, relative light units.

Table 1

Comparison of phenotypes in carriers and noncarriers of *LRP_{R611C}*. Means $\pm$ standard deviation are shown for quantitative traits. All kindred members with measured values were included for LDL, triglyceride, HDL, and BMI measurements. For blood pressure, fasting blood glucose, and diabetes, results for subjects over age 40 are shown.

Trait	<i>LRP_{R611C}</i> carriers	Noncarriers	<i>P</i> value
LDL (mg/dl)	170 $\pm$ 12	98 $\pm$ 5	6×10^{-6}
Triglycerides (mg/dl)	209 $\pm$ 71	68 $\pm$ 20	1×10^{-5}
HDL (mg/dl)	57 $\pm$ 8	56 $\pm$ 7	0.4
BMI (kg/m ²)	24.3 $\pm$ 2.6	24.4 $\pm$ 1.6	0.13
Systolic BP (mmHg)	168 $\pm$ 21	116 $\pm$ 5	8×10^{-5}
Diastolic BP (mmHg)	100 $\pm$ 14	81 $\pm$ 7	0.0025
Fasting blood glucose (mg/dl)	159 $\pm$ 43	80 $\pm$ 3	0.001
Diabetes (yes/no)	11/4	0/5	0.005